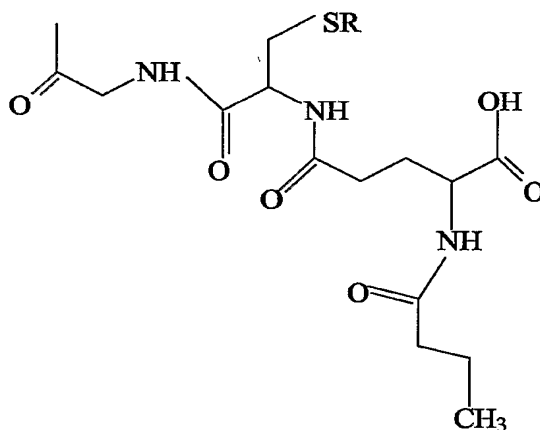1

GLUTATHIONE DERIVATIVES AND THEIR USES FOR THE TREATMENT OF VIRAL DISEASES

TECHNICAL FIELD

5   The present invention relates to glutathione derivatives of formula I:

10 and to their use as antiviral drugs, in particular for the treatment of Paramyxovirus, Orthomyxovirus, Herpes virus and HIV.

Glutathione (also known by the abbreviation GSH) is a
15 tripeptide (γ-glutamylcysteinylglycine) containing cysteine which is found in the eukaryotic cells in millimolar concentrations and has numerous functions in cellular physiology: it protects the cells from oxidative stress, maintaining the intracellular redox
20 state in reducing conditions, through metabolic interconversion, in its oxidized form of disulphide.

BACKGROUND ART

It is known and reported in numerous articles that
25 during viral infections there is a progressive reduction in GSH with consequent alteration of intracellular redox

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balance.

Unfortunately, GSH in vivo is oxidized rapidly, particularly in the presence of viral infections, a condition in which the redox state of the cells is unbalanced. Oxidized GSH is in turn reduced by the cellular glutathione reductase or eliminated from the cell through an ATP-dependent mechanism. Reduction of glutathione through cellular enzymes depends on the availability of reducing equivalents in the cell (NADH, NADPH) which are already depleted in the case of pathogen infection.

Recent data report, for example, that during infections in vitro with the Sendai parainfluenza 1 virus (SV), the Herpes Simplex-1 virus (HSV-1) and the Human Immunodeficiency Virus (HIV) a progressive decrease in GSH levels occurs. Many in vivo studies also report the existence of an unbalance of the redox state in the cells and body fluids of patients affected by HIV and Hepatitis C. Moreover, during experimental viral infections with the influenza virus a decrease in the anti-oxidant defenses and simultaneous increase in lipid oxidation products in the lungs and livers of animals sacrificed on the seventh day after infection have been described.

Many data suggest that an unbalance of the intracellular redox state is a key event in the replicative cycle of viruses, as it causes both an increase in replication and activation of nuclear transcription factors.

It is also known that the administration of reduced glutathione to infected cells prevents the decrease in intracellular GSH and inhibits viral replication in SV, HSV-1 and HIV infections, as reported, for example, in

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the article by Palamara, A. T., Perno, C. F., Ciriolo,
M. R., Dini, L., Balestra, E., D'Agostini, C. et al.
(1995) "Evidence for antiviral activity of glutathione:
in vitro inhibition of herpes simplex virus type 1
5   replication" in Antiviral Research 27, 237-253, and in
the article by Garaci, E., Palamara, A.T., Di Francesco,
P., Favalli, C., Ciriolo, M. R. & Rotilio, G. (1992),
"Glutathione inhibits replication and expression of
viral proteins in cultured cells infected with Sendai
10   virus" in Biochemical and biophysical research
communications 188, 1090-1096.

In experimental models the antiviral activity of GSH
seems to be correlated to an inhibition of the post-
15   transcriptional stages of virus replication, probably
preventing correct folding and maturation of specific
proteins.

The efficacy of anti-oxidant substances in viral
20   infections has been proven by in vivo studies, in
particular in the article by Palamara, A. T., Garaci,
E., Rotilio, G., Ciriolo, M. R., Casabianca, A.,
Fraternale, A. et al. (1996c) "Inhibition of murine AIDS
by reduced glutathione" in AIDS research and human
25   retroviruses 12, 1373-1381.

Administration of high doses of GSH reduces the viral
infection and inhibits advance of the disease, even, for
example, in a murine model of AIDS.
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However, one problem which occurs in the use and
administration of GSH, which is the problem addressed by
the present invention, is the fact that, although
antiviral activity of the antioxidant substances has
35   been clearly proven, it has also been proven that GSH is
not transported as such in the majority of cells or

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tissues.

DISCLOSURE OF INVENTION

For this reason it would be desirable to obtain
5   molecules which maintain the advantageous
characteristics of GSH, while at the same time allowing
the problem of transport to be solved, and which
therefore facilitate crossing the cellular membrane of
many types of cells.
10

According to the present invention, this problem is
solved by glutathione derivatives of formula I.

BRIEF DESCRIPTION OF THE DRAWINGS

15   The invention will now be described also with reference
to the accompanying figures, in which:
- Figure 1 shows the structural formula of a glutathione
derivative according to the present invention, that is,
n-butanoyl γ-glutamylcysteinylglycine (also known by the
20   abbreviation GSH-C4), together with its mass
spectrometry analysis after HPLC purification. The
analysis, conducted in negative ion mode, highlighted
the presence of a single mass peak 376.7 corresponding
to the monocharged molecule of interest ([M-H]⁻).
25   - Figure 2 shows the effect of GSH-C4 on Sendai virus
replication.
- Figure 3 shows the effects of GSH-C4 on HSV-1
replication.

30   According to the present invention, with the glutathione
derivatives (GSH) of formula I a strong antiviral
activity can be obtained in vitro, both against RNA
viruses (parainfluenza-1, Sendai), and DNA viruses
(Herpes Simplex, HSV-1), crossing the cellular membrane
35   both of MDCK cells and of Vero cells, and without
causing toxic effects on uninfected cells.

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GSH can be considered an antimicrobial agent which exercises its activity through different mechanisms depending on the host-pathogen system in question and on the concentration used.

The derivatives of the present invention are obtained by condensation of a carboxylic acid on the group $\mu-NH_2$ of the glutamic acid.

The compounds were synthesized in the laboratory using conventional solid phase peptide synthesis methods.

It was found that the butanoyl-glutathione according to the invention, indicated hereunder for convenience also with the abbreviation GSH-C4, acts with a different mechanism to the one shown for glutathione (GSH). In fact, besides significantly inhibiting HSV-1 replication, it is capable of preventing the cytopathic effects induced by the virus in Vero cells, and it also inhibits Sendai parainfluenza virus replication in MDCK cells, probably by interfering with essential cellular factors during the viral infection.

One of the advantages of the invention consists in the fact that GSH-C4 or a derivative thereof of formula I can preferably be used as a drug soluble in water, but also in cream or lotion form for the treatment of the Herpes Simplex 1 and 2 pathologies by topical administration.

Butanoyl glutathione of formula I can be considered an interesting antimicrobial agent against various pathogenic agents, as, according to one characteristic of the invention, it reduces both viral infectivity in the first stages of the disease, and viral production at

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a more advanced stage, an advantageous characteristic which can, at the present time, be considered unique.

BEST MODE FOR CARRYING OUT THE INVENTION

5   The invention is now described with reference to specific examples of embodiment and of tests of the derivative according to the invention, without the invention being intended as limited to said examples.

10 Example 1
Synthesis of glutathione derivatives (GSH-C2, GSH-C4, GSH-C6, GSH-C8, GSH-C12)

The compounds were synthesized in the laboratory using
15 conventional solid phase peptidic synthesis methods, in particular using the Fmoc (9-fluorenylmethoxycarbonyl) technique, suitably modified. It was then found that butanoyl glutathione GSH-C4 alone was able to solve the technical problem addressed by the present invention and
20 therefore the subsequent experiments were performed only on this derivative.

All laboratory syntheses were conducted with the manual technique using a suitable reaction container containing
25 a polystyrene resin (Wang-Gly-Fmoc resin produced by Novabiochem AG, Laufelfingen, Switzerland) functionalized with a glycine which has the N-terminal group protected by the Fmoc group.

30 The standard synthesis cycle comprised the phase of pretreatment of the resin by suspension in dichloromethane (Biosolve LTD, Netherlands) for one night, after which the Fmoc protection group was removed with piperidine (Fluka Chemie AG, Buchs, Switzerland) in
35 N,N-dimethylformamide (DMF) (Biosolve LTD, Netherlands) for 20 minutes.

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Simultaneously, 5 equivalents (eq.) of the appropriate Fmoc amino acid (Advanced Biotech Italia, Italy) were preactivated with 4.5 eq. of O-benzotriazol-1-
5   yl)1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (Advanced Biotech Italy), 5 eq. of N,N diisopropylethylamine, at the final concentration of 0.2 M in anhydrous N-methylpyrrolidone (Biosolve LTD, Netherlands). This solution was made to react with the
10 resin neutralized in situ for approximately 1 hour at 40°C.

Subsequently, a reaction is advisably performed with a 5% acetic anhydride solution (Fluka Chemie AG, Buchs,
15 Switzerland) in DMF to avoid undesired reactions of any amine groups that remain free.

Example 2
The butanoyl glutathione derivative (GSH-C4) was
20 prepared for treatment of the resin containing the peptidic portion with n-butanoic acid (Fluka Chemie AG, Buchs, Switzerland) previously activated as already described for the amino acids. The derivatives ethanoyl, exanoyl, octanoyl and dodecanoyl (GSH-C2, GSH-C6, GSH-C8
25 and GSH-C12) were prepared with similar methods, respectively using acetic anhydride and hexanoic, octanoic and dodecanoic acids (Fluka Chemie AG, Buchs, Switzerland) in place of butanoic acid.

30 Detachment of the synthesized compound from the resin and simultaneous removal of the side chain protector groups was conducted using a solution composed of trifluoroacetic acid (TFA) (Biosolve LTD, Netherlands), ethandithiol (FlukaChemie AG, Buchs, Switzerland), water
35 and triisopropylsilane (FlukaChemie AG, Buchs, Switzerland) in proportion 92.5:2.5:2.5:1 v/v for a time

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of 2 hours at room temperature. The acid solution was
vacuum concentrated to approximately 1 ml of final
volume and the final product precipitated with cold
diethyl ether and subsequently washed with the same
5   solvent.

All the molecules were purified by reverse phase liquid
chromatography (RP-HPLC) using a Waters C18 μBondapack
column, in which the solvent A was composed of 0.1% TFA
10 in water and the solvent B of the same percentage of
acid in acetonitrile. Elution of the compounds took
place with a gradient that started at 100% of solvent A
for 5 minutes, increased linearly up to 60% of solvent B
in 30 minutes and finally terminated with 100% of B in 5
15 minutes.

The fractions containing the molecules of interest were
collected, vacuum concentrated and then lyophilized. The
molecular weights of the glutathione derivatives were
20 confirmed by mass spectrometry analysis. The mass
spectra of each compound were acquired using an HP
Engine 5989-A spectrometer with a single quadruple
equipped with an electrospray source in negative ion
mode. All products were obtained with a final yield
25 varying from 75-78% and a purity of 95% after HPLC
analysis. An example of the mass spectra obtained is
shown in Figure 1.

Example 3
30 All the compounds obtained (GSH-C2, GSH-C4, GSH-C6, GSH-
C8, GSH-C12) were assayed on confluent monolayers of
uninfected Madin Darby canine kidney cells (MDKC) for
studies on the toxicity on the cells. The toxicity was
measured on the basis of microscopic examinations of
35 cellular morphology, of measurement of cellular vitality
after staining with Trypan blue and of the cell count.

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All compounds were diluted in RPMI, the final pH of the solution was approximately 7-7.3.

Example 4

5   Enzymatic determinations of the activity of glutathione peroxidase (E.C.1.11.1.9.) and glutathione S-transferase (E.C.2.5.1.18.) in hemolyzed human blood in the presence of GSH and of its derivative GSH-C4 were carried out according to the methods of Beutler (Beutler, E., 1984,

10 Red Cell Metabolism. A Manual of Biochemical Methods, 3rd edn. Grune & Stratton, New York). GSH and GSH-C4 were then oxidized by incubation at ambient temperature in the presence of 35% (v/v) H_2O_2 to respectively obtain GSSG and C4-GSSG-C4.

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Example 5

The entry of GSH and GSH-C4 into erythrocytes (RBCs) at 37°C in microcentrifugal test-tubes was measured using the oil-stop method. Erythrocyte suspensions with a 10%

20 hematocrit containing 5 mM of GSH or GSH-C4 and 10 mM GSH were incubated for 2h at 37°C. At different times (0, 5, 15, 30, 60 and 120 min), rates of 600 µl were stratified on 600 µl of bromododecane, centrifuged for 5 min at 10,000 rpm and the GSH and GSH-C4 content

25 measured in RBCs (Beutler, E., 1984, Red Cell Metabolism. A Manual of Biochemical Methods, 3rd edn. Grune & Stratton, New York).

Example 6

30 The stability of GSH-C4 in plasma was measured with the following procedure: GSH-C4 (1 mM) was incubated in human plasma at 37°C and for different incubation times (0, 15, 30, 60 and 120 min), rates of 200 µl were ultrafiltered using Amicon Centricon microconcentrators

35 by centrifugation at 2,000 rpm per 30 min. The filtered solution was then analyzed by high performance capillary

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electrophoresis (HPCE) to measure the content of GSH-C4 and C4-GSSG-C4.

Example 7

5 To measure the behavior of GSH-C4 derivatives in infected cells, Madin Darby canine kidney cells (MDCK) were grown in RPMI 1640 to which 5% of heat-decomplemented bovine fetal serum (Flow Laboratories, Italy) was added.

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The Senday virus (SV) belonging to the Paramyxovirus family is a virus with non-segmented single stranded RNA of negative polarity. This virus was reproduced by inoculation in the allantoic liquid of embryonated

15 chicken eggs. The monolayers of MDCK cells were infected with SV [3 hemagglutinating units (HAU) x 10^5 cells]. After incubation for 1 hour at 37°C (adsorption period), the unadsorbed viruses were removed, the monolayers were washed and then incubated in a complete medium

20 containing 2% of bovine fetal serum. The production of viruses by infected cells was determined in the cellular supernatants at different intervals of time from infection (p.i.), measuring the hemagglutinating activity towards type 0 Rh+ human erythrocytes (HAU),

25 according to standard procedures. To measure the antiviral activity, the compounds GSH-C2, GSH-C4, GSH-C6 were diluted in RPMI pH 7.3 and added at the desired concentration, immediately after the adsorption period of the virus. Monkey kidney cells (VERO) were grown in

30 RPMI, to which 5% of serum was added. The human herpes simplex virus type 1 (HSV-1), clinically isolated (TV1), was grown and titered in the Vero cells as described in Palamara, A. T., Perno, C. F., Ciriolo, M. R., Dini, L., Balestra, E., D'Agostini, C. et al. (1995). Evidence for

35 antiviral activity of glutathione: in vitro inhibition of herpes simplex virus type 1 replication. Antiviral

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Research 27, 237-253.

To obtain the viral infection, monolayers of Vero cells
were infected with HSV-1 at a MOI (multiplicity of
5   infection) of 0.03 PFU/cell. After incubation for 1 hour
at 37°C (adsorption period) the unadsorbed viruses were
removed, the monolayers were washed and then incubated
with culture medium containing 2% bovine fetal serum.
GSH-C4 was added in identical doses to the ones
10   indicated above immediately after the adsorption period
and was maintained in the culture medium until
completion of the experiments. Supernatants from
infected cells were collected at different times after
contact with the virus and tested for the ability to
15   form plaques in Vero cells, using a standard titration
method. Similar experiments were also conducted on HSV-1
TK-D305.

The results obtained on the basis of the examples
20   described are summarized below.

Toxicity of GSH derivatives

It was found that GSH-C4 and GSH-C2 derivatives do not
25   induce any toxic effect or change in cell morphology at
the concentrations used in the experiments conducted.
On the contrary, the addition of C8 (n-octanoyl) and C12
(n-dodecanoyl) derivatives caused marked toxic effects
and damaged the cells of the monolayers of uninfected
30   MDCK. The increase in these effects are dependent on the
dose administered and were observed starting from
concentrations of 0.1 mM. For this reason their activity
on viral replication was not taken into account.
Treatment of MDCK cells with Glutathione-C6 caused
35   damages on the uninfected monocellular layers
exclusively at the concentration of 10 mM. Some

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modifications to cellular morphology were found 24 hours after the addition of 2.5 and 5 mM. These substances cause inhibition of viral replication varying from 50% (2.5 mM) to 100% (10 mM). Due to the limited difference

5   between the cytotoxic doses and the antiviral doses, the GSH-C6 derivative was discarded as it was not optimal.

An inventive selection was then made between the various possible glutathione derivates in order to find which

10   ones could obtain the best effects as antiviral agents and it was surprisingly found that only with GSH-C4 derivatives according to formula I is it possible to obtain adequate antiviral efficacy and simultaneously solve the aforesaid problems correlated to the use of

15   GSH.

Metabolism of the GSH-C4 derivative

The enzymatic activities of glutathione peroxydase and

20   glutathione S-transferase were measured in hemolyzed human blood in the presence of GSH-C4 or GSH 2 mM as substrates. The activities of glutathione peroxydase and of glutathione S-transferase in the presence of GSH-C4 were 1.8% and 3.5% respectively, compared to those in

25   the presence of GSH. Moreover, GSH and GSH-C4 were chemically oxidized in the corresponding disulphide forms   (GSSG   and   C4-GSSG-C4).   The   activity   of glutathione reductase was measured on both substrates and the results obtained showed that the oxidized form

30   of C4-GSSG-C4 is gradually reduced with an enzymatic activity of 0.63 IU/g of hemoglobin (Hb), a Km of 50 mM and a Vmax equal to 12.6 μmol/min/mg Hb, while the oxidized form of GSH is reduced by glutathione reductase with an activity of 3.0 IU/g Hb, a Km 1.3 mM and a Vmax

35   6.3 μmol/min/mg Hb. These results show that once the oxidized dimer of GSH-C4 has formed, the cellular

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glutathione reductase is unable to efficiently reduce the compound.

Entry of GSH-C4 in the erythrocytes

Entry of GSH-C4 in the erythrocytes was measured and compared with GSH entry. The results obtained show that GSH-C4 crosses the membranes of the erythrocytes more rapidly than GSH, in this way solving the technical problem addressed by the present invention; in fact, the entry speed (calculated in the first 30 minutes) was 4.33 nmol/min/ml RBCs for GSH-C4 and 2.33 nmol/min/ml RBCs for GSH.

Effects of GSH-C4 on Sendai virus replication.

It was observed that the addition of GSH-C2 to MDCK cells infected with the Sendai virus caused a slight reduction in viral replication measured as hemagglutinating activity in the supernatant.

In particular, addition of the glutathione derivative at concentrations of 5.0 and 7.5 mM causes inhibition of the viral titer of 30% and 40% respectively.

Vice versa, the addition of GSH-C4 showed a much greater effect. The effect of GSH-C4 on reproduction of the Senday virus in MDCK cells is also shown in Figure 2.

MDCK cells were infected for 1 hour with 3 hemagglutinating units (HAU) x 10^5 cells. The cells were washed and then cultivated in the presence of different concentrations of GSH-C4 (range 0-7.5 mM) for 2 days. Production of the virus was assayed at 24 hours (panel A) and 48 hours (panel B) measuring the hemagglutinating activity on type 0 Rh+ human erythrocytes. Inhibition of

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Sendai virus replication is dependent on the dose of
GSH-C4 administered. A degree of inhibition varying from
88% (24h p.i.) to 93% (48h p.i.) was obtained in the
presence of 5 mM of GSH-C4. In the supernatant of
5   treated cells with composition containing GSH-C4 7.5 mM
no viruses were found. The dose of 7.5 mM of GSH-C4
therefore proved to be optimal as it is sufficient to
produce an excellent antiviral effect, without being
toxic for the cells, as also confirmed by microscopic
10   examination of the monolayers. 50% of inhibition of
viral reproduction at 48h (EC50) is obtained with GSH-C4
at a dose of only 3.6 mM, while a total of 7.6 mM of GSH
are required to obtain the same result.

15   Effect of GSH-C4 on HSV-1 replication

The effect of different doses of GSH-C4 on HSV-1
replication in VERO cells is shown in Figure 3, which
indicates the percentages of inhibition of replication
20   of the virus 48h after infection.

Vero cells were infected for 1 hour with HSV-1 at a MOI
of 0.03 PFU/cell. After repeated washings, GSH-C4 was
added at different concentrations (range 0-10 mM) to the
25   cell cultures. Replication of the viruses was tested in
the Vero cells 48h after infection using the plaque
technique. Figure 3 shows the results of four different
experiments which are concordant within 10% of the
values indicated.
30
The results obtained show that inhibition of virus
replication is dependent on the dose of GSH-C4
administered. A marked decrease (60% of inhibition
compared to the control) in viral replication was
35   obtained by adding 7.5 mM of GSH-C4. At the dose of 10
mM, no virus particles were detected in the cellular

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supernatant. The same inhibition was found 72h after infection.

The addition of GSH at a concentration of 10 mM does not
5   induce complete inhibition of the virus and only produces a reduction in virus replication of around 2.5 log. Moreover, GSH-C4 protects the Vero cells from the cytopathic effects induced by the virus and does not induce any toxic effects in uninfected Vero cells. The
10   effect of GSH-C4 was also assessed on a strain of defective virus for thymidine kinase (Δ305).
Vero cells were infected for 1h at 37°C with the strain Δ305 and then kept in culture in the presence of different concentrations of GSH-C4. The production of
15   viruses was measured 24, 48 and 72h after infection by assay of the plaque forming units.

The table shows the results of one experiment representative of three. The variability between results
20   obtained in the various experiments did not exceed 10%.

Tab. 1 Effects of GSH-C4 on HSV-1 TK-

	HSV-1 TK- (pfu/ml)		
	24h	48h	72h
Ctr.	1.38×10^5	1.96×10^5	2.56×10^6
5 mM	2.59×10^5	2.42×10^6	2.13×10^6
7.5 mM	1.59×10^5	2.56×10^5	4.33×10^5
10 mM	1.53×10^5	2.6×10^5	3.44×10^5

25   The results obtained show that GSH-C4 is less active against the strain TK⁻ compared with when it is used on the wild type strain. In fact a significant inhibition of virus titer (approximately 1 log) is observed only at a concentration of 7.5 mM 72h after infection.

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Finally, it is apparent that as it has been proven
experimentally that the derivatives according to the
present invention are efficacious in the treatment of
5   diseases deriving from the Sendai virus and therefore of
paramyxoviruses, they are also efficacious against
orthomyxoviruses. Moreover, as all the tests performed
are concordant in confirming efficacy against various
viruses, it is obvious to infer that GSH-C4 derivatives
10   of formula I are antiviral agents.

Finally, although the examples are relative to the GSH-
C4 derivative in which the only SH group is not
substituted, it is legitimate to assume that all the
15   derivatives in which the SH group of GSH-C4 according to
the present invention is substituted with protection
groups are equally efficacious in antiviral treatments.